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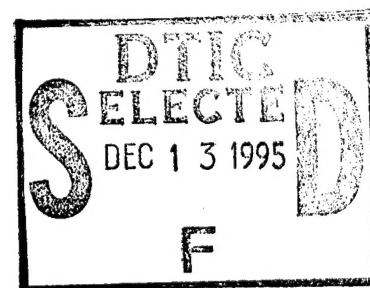
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## INTRODUCTION

Breast cancer is the most common malignancy among women from developed nations. In the United States, it is responsible for 46,000 deaths annually. When initially diagnosed, many breast tumors express receptors for the steroid hormone estrogen and are amenable to treatment with antiestrogenic drugs. An unfortunate side effect of such treatment is that tumors may subsequently recur and possess a phenotype that is resistant to further anti-hormone treatment. These estrogen independent tumors often overexpress genes involved in the regulation of cell growth.

One such gene is the transmembrane tyrosine kinase c-erbB-2. C-erbB-2 is 185 kDa protein that shares homology with epidermal growth factor receptor (EGFR) and the related c-erbB-3 and c-erbB-4 genes, although no ligand specific for c-erbB-2 has been isolated. Overexpression of c-erbB-2 in human breast cancer correlates with loss of estrogen receptor (ER), poor prognosis, poor response to endocrine therapy, and earlier death (1). Our laboratory has investigated the role of c-erbB-2 in breast cancer growth and progression by transfection of the c-erbB-2 cDNA into estrogen receptor positive, poorly tumorigenic MCF-7 cells. We found that cells overexpressing the protein, denoted MB3 cells, formed small tumors in nude mice in the absence of estrogen, but that these tumors were not metastatic and could still be stimulated by estrogen. The size of the tumors seemed to correlate the degree to which the transfected receptor was constitutively phosphorylated on tyrosine residues (2). We therefore hypothesized that the critical factor responsible for tumorigenicity was the level of constitutive phosphorylation of the receptor.

We proposed to transfect MCF-7 cells with a mutant c-erbB-2 gene carrying a valine to glutamic acid conversion in the transmembrane region, resulting in a constitutively active kinase (3,4). This approach would address the question of whether the limited tumorigenicity of wild type c-erbB-2 overexpressing cells was due to an insufficiently high level of kinase activity, or because overexpression of this protein alone was insufficient to result in invasive, metastatic growth of the cancer.

## BODY

In our Statement of Work, we outlined three specific Tasks to be accomplished in the course of our investigation. To achieve these goals, we stated three Technical Objectives in the body of our proposal. Below we restate each of these Tasks and Technical Objectives, and discuss our progress in reaching each of them.

### **Task 1: Construction of a vector directing expression of mutant c-erbB-2.**

*Technical objective #1: We will transfect ML-20 cells with an expression vector directing high levels of expression of a constitutively active c-erbB-2 gene.* Detection of micrometastases from mice bearing MB3-derived tumors proved difficult and previous attempts to transfect MB3 cells with *lacZ* were unsuccessful. We therefore chose MCF-7 cells that already stably express the bacterial  $\beta$ -galactosidase gene, denoted ML-20 cells (5), as recipients for a second transfection of wild type and mutant c-erbB-2. Because ML-20 cells are hygromycin resistant as a result of the *lacZ* transfection, we first constructed a eukaryotic expression vector containing the c-erbB-2 cDNA that would confer resistance to a different selectable marker.

We chose the vector pCHisC, a double transcription vector that confers resistance to the drug l-histidinol (Figure 1). In our initial cloning strategy, we proposed to exchange a 3.4 kb *Stu* I fragment between our wild type c-erbB-2 4.5 kb cDNA and a cDNA containing the valine to



glutamic acid mutation at position 659 obtained from Dr. Paolo Di Fiore at the NCI (6). We later discovered that the cDNA from Dr. Di Fiore lacked the second Stu I site in the 3' untranslated region. We therefore decided to exchange a 1.8 kb Eco RI fragment, which also encompass the transmembrane region containing the mutation, between the two c-erbB-2 cDNA clones; however, we had to first remove an Eco RI site in the polylinker of our expression plasmid (see Fig. 1). We digested pCHisC with Eco RI, isolated the linearized fragment, filled in the overhangs with the Klenow fragment of DNA polymerase I, and re-circularized the blunt ends of the vector. Removal of the Eco RI site was demonstrated by the resistance of the plasmid to Eco RI digestion. The plasmid was next digested with Xba I and ligated to the 4.5 kb c-erbB-2 cDNA previously described to generate pCHisCerbB2(wt). Finally, this plasmid was digested with Eco RI, purified, and ligated to a 1.8 kb Eco RI fragment containing the point mutation derived from the plasmid LTR/erbB-2 Glu to generate pCHisCerbB2(Glu). The identity of both wild type and mutant c-erbB-2 plasmids was confirmed by dideoxy sequencing of the transmembrane region (data not shown). *The cloning of the wild type and mutant c-erbB-2 genes successfully fulfilled the goal of Task 1.*

#### **Task 2: Transfection of ER+ breast cancer cells with the mutant c-erbB-2**

ML-20 cells were transfected with vector control, wild type, or mutant c-erbB-2 by the method of calcium phosphate precipitation and single drug-resistant clones isolated and screened for expression of c-erbB-2 protein by flow cytometry. Approximately 35 wild type (designated MLV, because they are derived from ML-20 cells and express a c-erbB-2 protein with a valine at position 659) and 35 mutant c-erbB-2 (designated MLE, because of the glutamic acid, or E in single letter amino acid code) clones, as well as 10 control transfectants, were isolated. *This transfection and selection of clones meets to goals set forth in Task 2a.* Because we have previously observed loss of wild type c-erbB-2 expression in the presence of estrogen (2), we screened for protein expression in both IMEM plus 5% fetal calf serum (FCS) and under estrogen-free condition of phenol red-free IMEM plus 5% charcoal stripped calf serum (CCS). As expected, a number of MLV clones were positive for protein expression by flow cytometry when grown in CCS but not in FCS. Protein levels approached or exceeded those of our previous c-erbB-2 transfected MCF-7 cells, MB3 (Figure 2, panels A-G). We stained these new clones to determine the expression of  $\beta$ -galactosidase and found that 90-95% of the cells become blue. The goal of generating wild type c-erbB-2 expressing cells that stain blue, thereby allowing us to more readily detect micrometastases, was accomplished by the generation of these cell lines. *This characterization of the wild type c-erbB-2 transfectants partially fulfills the goals described in Task 2b.*

In contrast to wild type c-erbB-2 transfectants, no MLE clones overexpressed the protein in FCS, and, surprisingly, only one did in CCS (Figure 2, panel H). With this clone, levels of expression were lower and more heterogeneous than seen with wild type c-erbB-2, and this clone ceased to grow in culture after a few weeks. This finding led us to hypothesize that overexpression of a constitutively active c-erbB-2 was selected against *in vitro*. We decided that an alternative cloning strategy would be necessary to study the effect of mutant c-erbB-2 overexpression.

*Technical objective #2: We will determine the biological effect of overexpression of mutant c-erbB-2 in ML-20 cells in vitro and in vivo.* While we tried to work out conditions under which we could express the constitutively active mutant receptor, we began to characterize the MLV transfectants. Flow cytometry indicated that the levels of protein expression in our transfectants were near those seen in MB3 transfectants. Because the size of tumors formed in nude mice by MB3 cells correlated

with the degree of constitutively active kinase (2), we wished to determine whether the levels of kinase activity in our new erbB-2(wt) transfectants were near that of the MB3 cells.

Western analysis of whole cell lysates with an anti-erbB-2 antibody confirmed the flow cytometric data and demonstrated that protein levels were near or above those of MB3 cells (Figure 3A). An anti-phosphotyrosine antibody (Figure 3B) demonstrated that levels of constitutively active kinase were dramatically higher in the erbB-2(wt) cells than in MB3 cells. It is possible this comparison reflects loss of kinase activity by MB3 cells over time in culture, although no loss in cell surface protein has been seen. Alternatively, MLV transfectants may have dramatically higher levels of active c-erbB-2. We have now thawed MB3 cells and will compare the behavior of the newly thawed cells with those in culture for an extended period. If it is the case that the MLV transfectants do have much higher kinase activity than the MB3 cells, it implies that overexpression of the unaltered form of the protein may be sufficient to result in constitutive activation, provided that absolute levels of the protein exceed a certain threshold. In the future, we will compare the behavior of these newly generated transfectants with MB3 cells to determine the phenotypic effect of such a high level of kinase activity. *The results described here partially fulfill the goals set forth in Task 2b. Experiments are just beginning to characterize MLV transfectants in vitro, as described in Task 2c.*

**Task 3: Examination of expression of transfected erbB-2 in the presence and absence of estrogen.**

*Technical objective #3: We will determine the stability of overexpression of the mutant c-erbB-2 gene under estrogen-containing and estrogen-free conditions.* Characterization of wild type c-erbB-2 expression has reproduced the same results seen in our previous c-erbB-2 transfection. Cells grown in FCS fails to stably overexpress the protein while cells in CCS express high levels. We have thus far been unable to stably overexpress the mutant c-erbB-2 gene under any conditions, and have therefore been unable to address many of the questions regarding expression of the mutant gene.

Only one clone of MLE cells stained positive for c-erbB-2 expression by flow cytometry (Figure 2H), and this clone ceased to grow after a brief time in culture. When examined microscopically, these cells exhibited a phenotype typified by large vacuoles in the cells and loose attachment to the flask, and the cells grew very slowly, ultimately falling off the dish, dead. These observations led us to hypothesize that constitutive overexpression of the erbB-2(Glu) might induce apoptosis in these cells. In our original proposal, we indicated that problems might arise when we attempted to overexpress this constitutively active protein, and proposed use of an inducible gene expression system to overcome any obstacles. We have therefore tried to establish conditions under which we could express erbB-2 (Glu) in an inducible manner to allow us to better understand the effect its expression was having on the cells.

We chose to utilize a recently described system in which gene expression is under the control of a promoter activated by a transactivator whose activity is repressed by the presence of tetracycline (Tc) (7). We chose this system because it has been reported to exhibit low levels of basal activity and a high degree of induction; furthermore, the inducing agent, tetracycline, has no effect on mammalian cells in the concentration required for gene regulation. In these respects this system is superior to others in which high backgrounds are observed or where potentially toxic agents must be used to induce gene expression. ML-20 cells were transfected with the tetracycline transactivator



(Tta) gene and we isolated a number of stable clones. We screened these clones for the ability to induce expression of a luciferase reporter construct downstream of the tetracycline responsive promoter in a transient transfection assay. One clone was able to induce expression of the reporter gene approximately 500-fold, and was chosen as the recipient for the mutant c-erbB-2 transfection. The 4.5 kb erbB-2(Glu) cDNA was excised from the pCHisCerbB2(Glu) vector by Xba I digestion, ligated into the pUHD10.3 plasmid downstream of the tetracycline responsive promoter (7), and transfected into the Tta-expressing ML-20 cells. Drug selection of the cells was carried out in the presence of 100 ng/ml anhydrotetracycline (ATc), an analog of tetracycline with a 10-fold higher affinity for the Tta transactivator (8), to ensure that the erbB-2(Glu) was not expressed.

Approximately 20 drug-resistant colonies were assayed for the ability to induce erbB-2(Glu) expression upon withdrawal of ATc. Although most reports indicate that expression of the gene of interest can be seen within 24 to 48 hours of Tc or ATc withdrawal, we found that longer periods of ATc withdrawal were required to see c-erbB-2 induction. We screened clones in both FCS and CCS, because we have seen an inability to overexpress erbB-2(wt) in FCS. After 7 days of ATc withdrawal, we were able to identify a clone that induced expression of the mutant c-erbB-2 gene in both FCS and CCS to levels seen in SK-Br-3 cells, an ER- cell line with an amplified c-erbB-2 gene. This result has now been seen in two other clones, although the level of induction is not as high. We are now in the process of characterizing the effect on the cells of overexpression of the mutant protein, and trying to determine whether it results in apoptosis. *These results partially fulfill the goals of Task 2c, and of Task 3a.*

## CONCLUSIONS

At the end of the first year of this study, we have completed the goals outlined in Task 1. We have successfully cloned the wild type and mutant genes into the appropriate expression vectors, and have isolated cell lines that stable express the wild type c-erbB-2 and the *lacZ* gene. Generation of cell lines stably overexpressing the wild type protein partially fulfills the goals of Tasks 2 and 3. While we have been unable to overexpress the mutant gene in a similar manner, we have stably transfected the mutant receptor into cells utilizing a system that allows us to induce gene expression. These cells will be used to fulfill the remainder of the goals set forth in Tasks 2 and 3.

It now appears possible that constitutive overexpression of the mutant c-erbB-2 gene may be detrimental to the cells and induce apoptosis. If this is the case, it is unlikely that these cells would be more tumorigenic in nude mice than the original MB3 transfectants. However, the high levels of constitutive kinase activity observed in MLV transfectants relative to MB3 cells allows us the option to compare the tumorigenicity of these cells with MB3 cells. If our original hypothesis is correct, and tumorigenicity is related to levels of constitutive kinase activity, then the MLV transfectants may prove to be a useful tool for approaching this problem.

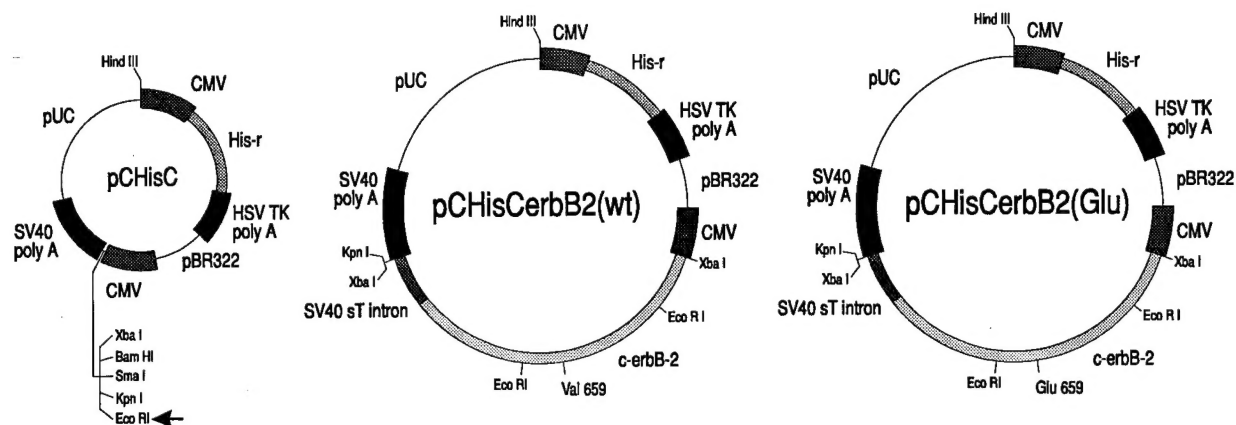
It has recently been reported that overexpression of a constitutively active c-erbB-2 gene in mammary epithelial cells results in apoptosis upon serum withdrawal, and that this response can be blocked by glucocorticoids but not peptide growth factors (9). If we determine that our erbB-2(Glu) overexpressing MCF-7 cells are undergoing apoptosis as a result of overexpression of this protein, we will attempt to dissect the pathways through which this response is mediated.

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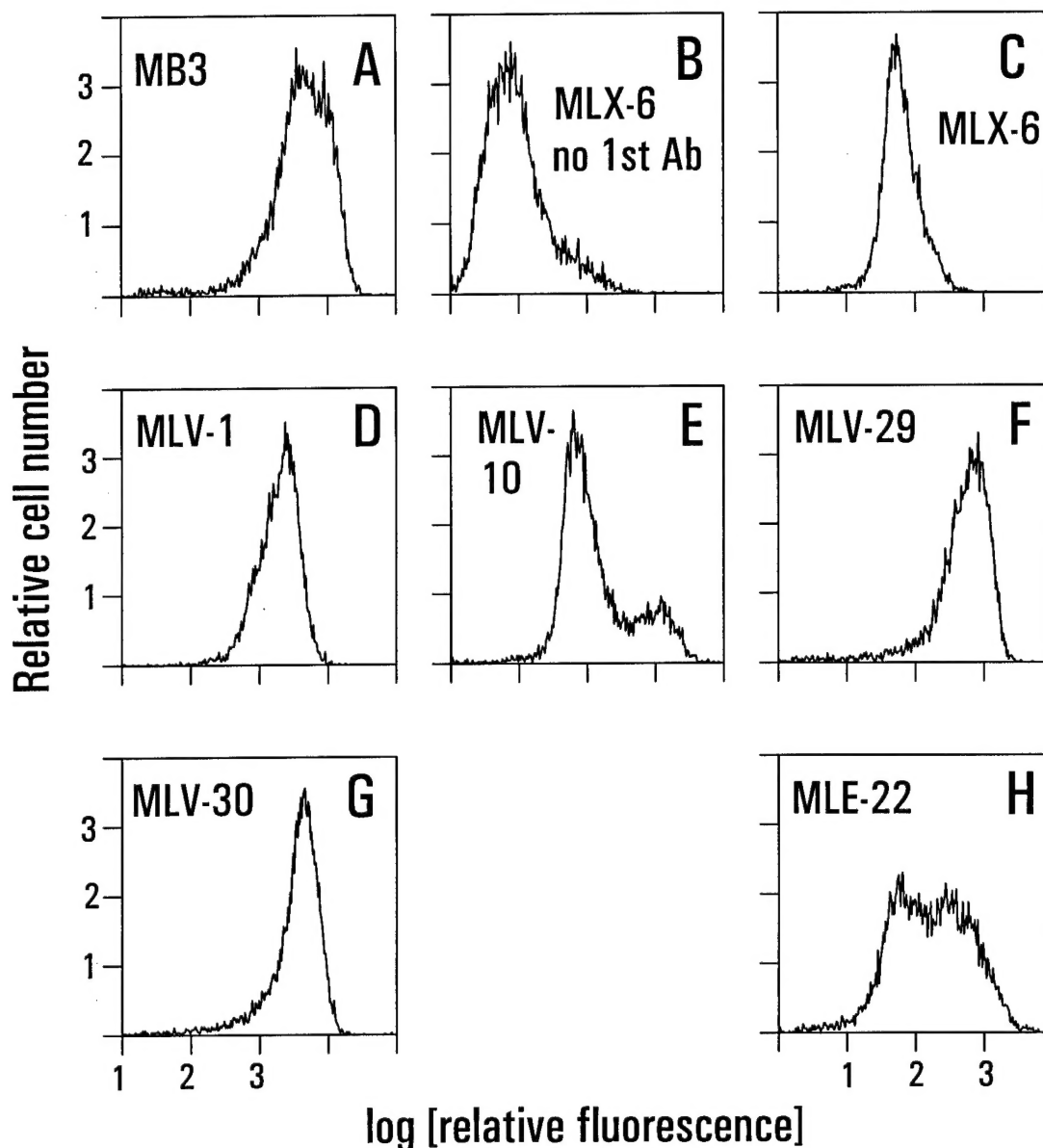
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# **APPENDIX**

## **Figures and Legends**

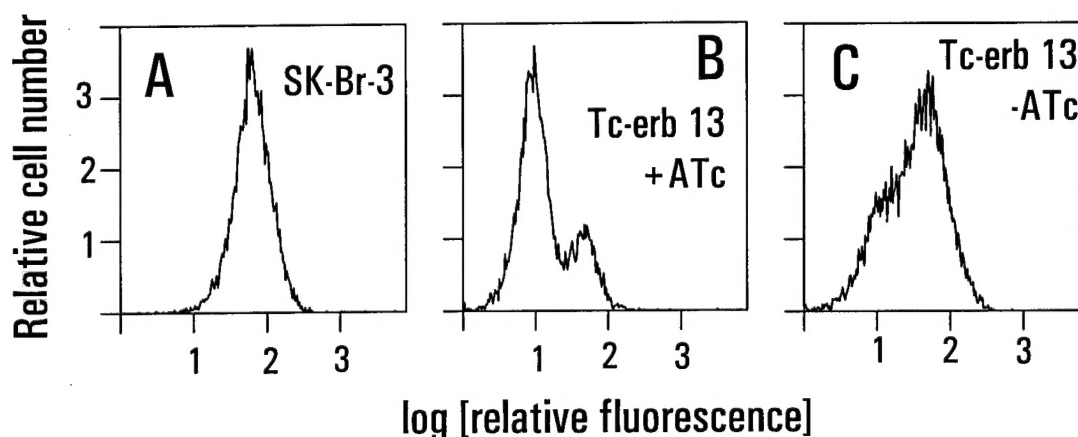
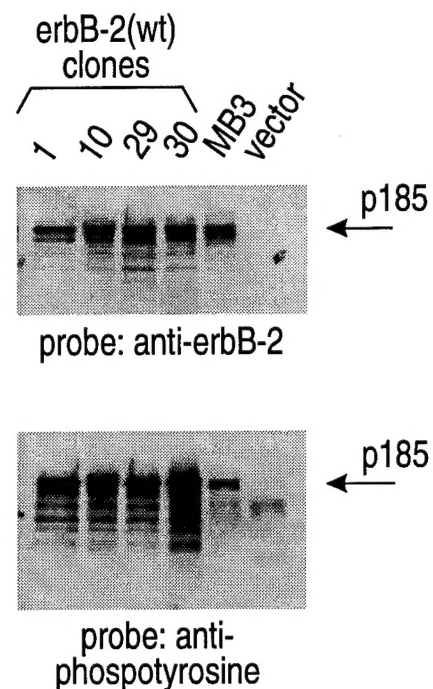


**Figure 1. Double transcription eukaryotic expression plasmids used in this study.** The plasmid pCHisC contains two cytomegalovirus immediate early gene promoters to drive transcription. The first unit confers resistance to the drug l-histidinol and the second promoter is located upstream of a polylinker. To construct wild type and mutant c-erbB-2 expression plasmids, the Eco RI site in the pCHisC polylinker (arrowhead) was first removed as described in the text. The 4.5 kb c-erbB-2 cDNA was excised from the plasmid pCNCerbB2 (2) with Xba I and ligated into pCHisC to generate pCHisCerbB2(wt). Subsequently, a 1.8 kb Eco RI fragment was exchanged between pCHisC-erbB2(wt) and LTR/erbB2-Glu (6) to generate pCHisCerbB2(Glu).



**Figure 2. Flow cytometric analysis of c-erbB-2 transfected ML-20 cells.** ML-20 cells were transfected with the vectors shown in Figure 1, drug selected in FCS, and expanded in CCS before screening for expression of the transfected c-erbB-2 gene by flow cytometry. Cells were stained with an anti-erbB2 primary mouse monoclonal antibody and a secondary anti-mouse antibody coupled to fluorescein isothiocyanate. Relative fluorescence was determined using a FACStar Plus (Becton Dickinson). The X axis denotes relative fluorescence in log units, while the Y axis shows relative cell number. Panel A, previously described erbB-2 transfected MCF-7 cells, denoted MB3; Panel B, unstained control transfectants; Panel C, control transfectants stained with the anti-erbB-2 antibody; Panels D, E, F, G, various erbB-2(wt) clones that stained positive for protein expression; Panel H, the single clone that stained positive for expression of the mutant erbB-2(Glu) protein.

**Figure 3. Western analysis of erbB-2(wt) transfectants.** Wild type c-erbB-2 transfectants were grown in CCS and whole cell lysates prepared. 50  $\mu$ g total cellular protein was run on a 4-20% tris-glycine gradient gel (Novex), transferred to nitrocellulose, and probed using an anti-erbB-2 monoclonal antibody (Panel A). A duplicate gel was run and probed with an anti-phosphotyrosine antibody (Panel B). MB3 and vector only control transfectants are included for comparison. Numbers above the lanes refer to MLV clone numbers. Numbers above the lanes refer to MLV clone numbers.



**Figure 4. Inducible expression of the erbB2(Glu) mutant upon withdrawal of anhydrotetracycline.** Cells transfected with the tetracycline transactivator (Tta) and mutant erbB2(Glu) under control of the tetracycline responsive promoter were grown in the presence or absence of tetracycline and analyzed for expression of c-erbB-2 by flow cytometry. Panel A, SK-Br-3 cells, which overexpress c-erbB-2 as a result of gene amplification; Panel B, mutant erbB2 transfected cells grown in the presence of anhydrotetracycline (ATc), Panel C, mutant erbB2 transfected cells grown in the absence of ATc for 7 days.